

KIR9601TR.SEQ  
HL9  
DEB/CSE/HL

PATENT APPLICATION  
Docket No.: KIR96-01

08/602272



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Michael J. Elliott, Ravinder N. Maini  
and Marc Feldmann

Title: METHODS OF PREVENTING OR TREATING  
CARDIOVASCULAR, CEREBROVASCULAR AND  
THROMBOTIC DISORDERS WITH TUMOR NECROSIS  
FACTOR ANTAGONISTS

"EXPRESS MAIL" Label No. <u>EG978844959US</u>	
I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 and is addressed to Assistant Commissioner for Patents, Washington, D.C. 20231 on	
<u>February 16, 1996</u> Date	<u>Mariamne L. Boothby</u> Signature
<u>Mariamne L. Boothby</u> Typed or printed name of person signing certificate	

TRANSMITTAL OF SEQUENCE LISTING IN COMPUTER READABLE FORM  
IN COMPLIANCE WITH 37 C.F.R. §§1.821(e) AND (f)

Box Sequence  
Assistant Commissioner for Patents  
Washington, DC 20231

Sir:

Transmitted herewith is a copy of the "Sequence Listing" in computer readable form as required by 37 C.F.R. §1.821(e). As required by 37 C.F.R. §1.821(f), Applicants' Agent hereby states that the content of the "Sequence Listing" in paper form and of the computer readable form of the "Sequence Listing" are the same.

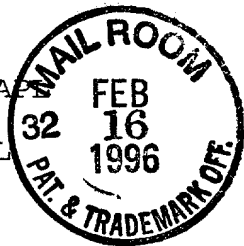
Respectfully submitted,

Helen Lee

Helen Lee  
Agent for Applicants  
Registration No. 39,270  
Telephone: (617) 861-6240

Lexington, Massachusetts 02173

Date: February 16, 1996



METHODS OF PREVENTING OR TREATING CARDIOVASCULAR,  
CEREBROVASCULAR AND THROMBOTIC DISORDERS  
WITH TUMOR NECROSIS FACTOR ANTAGONISTS

Description

5 Background of the Invention

Platelets and fibrinogen play integral roles in the formation of blood clots (thrombi). Platelets first adhere to macromolecules in the subendothelial regions of an injured blood vessel; they then aggregate to form the nidus  
10 of a thrombus. Platelet aggregation is mediated by fibrinogen binding to the activated platelet membrane glycoprotein IIb/IIIa (GP IIb/IIIa) receptor. The platelets stimulate local activation of plasma coagulation factors, which leads to the conversion of fibrinogen bound  
15 to GP IIa/IIIb receptor to fibrin monomers which polymerize to form the matrix (fibrin clot) of a thrombus.

Epidemiological studies have shown that elevated levels of plasma fibrinogen are associated with acute myocardial infarction, ischemic heart disease, coronary  
20 mortality, stroke in men, deep vein thrombosis and thrombophlebitis (Meade et al., *Eur. Heart J.* 16 Suppl A:31-35 (1995); Meade et al., *Br. Med. Bull.* 33:283-288 (1977); Meade et al., *Lancet* ii:533-537 (1986); Wilhelmsen et al., *N. Engl. J. Med.* 311:501-505 (1984); Kannel et al.,  
25 *JAMA* 258:1183-1186 (1987); Stone and Thorp, *J. R. Coll. Gen. Pract.* 35:565-569 (1985); Balleisen et al., *Lancet* ii:461 (1987); Lee et al., *J. Clin. Epidemiol.* 43:913-919 (1990); Moller and Kristensen, *Arterioscler. Thromb.* 11:344-350 (1991); Broadhurst et al., *Atherosclerosis*  
30 85:169-173 (1990); Handa et al., *Atherosclerosis* 77:209-213 (1989)).

Increased levels of platelet GP IIa/IIIb receptors are

also associated with acute myocardial infarction (Brown et al., *Eur. J. Clin. Invest.* 24 Suppl. 1:9-15 (1994); Giles et al., *Eur. J. Clin. Invest.* 24:69-72 (1994)).

Platelets play an important role in the development of the atherosclerotic lesions that typically underlie coronary artery disease, aortic aneurysm, arterial disease of the lower extremities and cerebrovascular disease.

Thus, elevated circulating fibrinogen and/or platelet levels are independent risk factors that are at least as significant as cholesterol or hypertension for vascular diseases, such as coronary heart disease and cerebrovascular disease.

#### Summary of the Invention

The present invention is based on the surprising discovery that inhibition of the biological activity of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) reduces fibrinogen and platelet levels in individuals with active rheumatoid arthritis. As a result of Applicants' discovery, a method is provided herein to treat and/or prevent a cardiovascular and/or a cerebrovascular disorder in an individual comprising administering a therapeutically effective amount of a tumor necrosis factor (TNF) antagonist to the individual.

In a second embodiment, the invention relates to a method for treating and/or preventing a thrombotic disorder in an individual comprising administering a therapeutically effective amount of a TNF antagonist to the individual.

In another embodiment, the present invention relates to a method of decreasing plasma fibrinogen in an individual comprising administering a therapeutically effective amount of a TNF antagonist to the individual.

The present invention further relates to a method of decreasing platelet levels and/or platelet aggregation in an individual comprising administering a therapeutically

03603272 00000000

effective amount of a TNF antagonist to the individual.

TNF antagonists useful in the methods of the present invention include anti-TNF antibodies and receptor molecules which bind specifically to TNF, compounds which prevent and/or inhibit TNF synthesis or TNF release, such as thalidomide, phosphodiesterase inhibitors (e.g., pentoxifylline and rolipram), tenidap, A2b adenosine receptor agonists and A2b adenosine receptor enhancers and compounds which prevent and/or inhibit TNF receptor signalling.

#### Brief Description of the Drawings

Figures 1A-1B are graphic representations of results showing the effect of  $\text{TNF}\alpha$  on circulating interleukin-6 (IL-6). Figure 1A shows a detailed time/response profile on day 0 and 1, with the mean sampling times indicated. Figure 1B shows changes in circulating IL-6 in the same three patient groups over the longer term.

Figure 2 is a graphic representation of results showing the effect of  $\text{TNF}\alpha$  blockade on circulating serum amyloid A (SAA).

Figure 3 is a graphic representation of results showing the rate of change in circulation IL-6 and acute phase proteins.

Figures 4A-4B are scatter graph representations showing the relationship between circulating IL-6 and C reactive protein (CRP). Figure 4A shows the relationship between circulating IL-6 and CRP in all 73 patients pre-treatment (Spearman's rank correlation coefficient ( $\rho=0.55$ ,  $p<0.002$ )). Figure 4B shows the relationship between the reduction in circulating IL-6 by day 3 and the reduction in CRP over the same period in all 73 patients ( $\rho=0.59$ ,  $p<0.002$ ).

Detailed Description of the Invention

Many patients with rheumatoid arthritis (RA) ultimately die from cardiovascular and cerebrovascular diseases. Although other factors undoubtedly also  
5 contribute to the excess cardiovascular and cerebrovascular mortality seen in RA (Wolfe *et al.*, *Arthritis Rheum.* 37:481-494 (1994)), persistently elevated plasma fibrinogen and/or platelet levels are major contributors to these diseases.

10 Platelets and fibrinogen play integral roles in the formation of blood clots (thrombi). Typically, the formation of a blood clot begins with the adhesion of platelets to macromolecules in the subendothelial regions of the injured blood vessel. The adhesion of platelets is  
15 mediated by platelet surface receptors which bind to extracellular matrix proteins in the exposed subendothelium, such as von Willebrand factor, collagen, fibronectin, vitronectin and laminin. Platelet adhesion results in a monolayer of platelets. Platelet activation  
20 subsequently occurs in response to agonists such as epinephrine, adenosine diphosphate (ADP), collagen and thrombin. Activation leads to the exposure of GP IIb/IIIa receptors on the platelet surface. GP IIb/IIIa receptors on activated platelets are then available to bind adhesive  
25 proteins, typically fibrinogen. Subsequently, platelets aggregate to form the primary hemostatic plug.

Platelet aggregation is mediated, at least in part, by the cross-linking by fibrinogen of GP IIb/IIIa receptors on adjacent platelet membranes. Fibrinogen bound to the GP  
30 IIb/IIIa receptors is cleaved by thrombin into fibrin monomers which then polymerize to form the matrix of the blood clot. Formation of the fibrin meshwork (fibrin clot) results in reinforcement and stabilization of the platelet plug at the site of vascular disruption.

35 Under normal circumstances, blood clots serve to

prevent the escape of blood cells from the vascular system,  
a desirable effect upon incurring a blood vessel wound.  
However, during certain disease states, clots can restrict  
or totally occlude blood flow resulting in cellular  
5 necrosis.

The work described herein clearly shows the surprising  
result that TNF $\alpha$  blockade in the short term leads to  
normalisation of fibrinogen levels in many patients and  
suggests that fibrinogen levels can be controlled through  
10 effective long term TNF blockade. The surprising result  
that TNF $\alpha$  blockade in the short term leads to normalisation  
of platelet levels was described in U.S. Application No.  
08/324,799 (filed October 18, 1994); Elliott et al.,  
*Arthritis Rheum.* 36:1681-1690 (1993); and Elliott et al.,  
15 *Lancet* 344:1105-1110 (1994), which references are entirely  
incorporated herein by reference.

Reducing plasma fibrinogen and platelet levels reduces  
the risk of developing many cardiovascular, cerebrovascular  
and thrombotic disorders. Thus, the development or  
20 progression of these disorders can be prevented or  
decreased with long term blockade of TNF. Cardiovascular,  
cerebrovascular and thrombotic disorders can also be  
treated with long term blockade of TNF.

The present invention is directed to a method for  
25 treating and/or preventing a cardiovascular and/or a  
cerebrovascular disorder in an individual. The method  
comprises administering a therapeutically effective amount  
of a TNF antagonist to the individual. As used herein, a  
"cardiovascular disorder" includes acute <sup>myocardial</sup> myocardial  
9 infarction, and cardiac thrombotic disorders, such as deep  
30 vein thrombosis and thrombophlebitis. As used herein, a  
"cerebrovascular disorder" includes vascular disorders  
related to the brain, such as stroke.

The present invention is also directed to a method of  
35 treating and/or preventing a thrombotic disorder in an

individual comprising administering a therapeutically effective amount of a TNF antagonist to the individual. As used herein, a "thrombotic disorder" is a condition where thrombosis is a pathogenic component. For example, a

9 5 "thrombotic disorder" includes thromboembolic disorders (e.g., pulmonary thromboembolism), ischemic events (e.g., transient ischemic attack), stroke, acute <sup>myocardial</sup> ~~myocardial~~ infarction, deep vein thrombosis and thrombophlebitis.

10 The present invention further relates to a method of decreasing plasma fibrinogen in an individual comprising administering a therapeutically effective amount of a TNF antagonist to the individual. The TNF antagonist binds with high affinity to TNF $\alpha$  in the individual, resulting in a reduction in plasma fibrinogen in the individual. For  
15 example, as shown in the Example, administration of the antibody chimeric A2 (cA2) to 49 patients resulted in a significant reduction in plasma fibrinogen in many of the patients.

20 The present invention can be used to treat and/or prevent thrombosis. For example, the present invention can be used to prevent thrombosis in pulmonary embolism, ischemic events (e.g., transient ischemic attack), deep vein thrombosis, coronary bypass surgery, surgery to insert a prosthetic valve or vessel (e.g., in autologous, non-  
25 autologous or synthetic vessel graft) or deployment of a vascular (coronary or peripheral) stent. The invention can also be used to treat and/or prevent occlusion, reocclusion, stenosis and/or restenosis of blood vessels. For example, the invention can be used to treat and/or  
30 prevent recurrence of cardiovascular, cerebrovascular and/or thrombotic disorders.

TNF $\alpha$ , a pleiotropic cytokine released by activated T cells and macrophages, is expressed as a mature 17 kDa protein that is active as a trimer (Smith, R.A. and  
35 Baglioni, C., *J. Biol. Chem.* 262: 6951-6954 (1987)).

Trimeric cytokines such as TNF $\alpha$  and the closely related protein lymphotoxin (TNF $\beta$ ), exert their biological activity by aggregating their cell surface receptors. The TNF trimer binds the receptors on the cell surface causing

5 localized crosslinking of TNF receptors into clusters necessary for signal transduction. As used herein, a "tumor necrosis factor antagonist" decreases, blocks, inhibits, abrogates or interferes with TNF activity *in vivo*. For example, a suitable TNF antagonist can bind TNF

10 and includes anti-TNF antibodies and receptor molecules which bind specifically to TNF. A suitable TNF antagonist can also prevent or inhibit TNF synthesis and/or TNF release and includes compounds such as thalidomide, tenidap, and phosphodiesterase inhibitors, such as, but not

15 limited to, pentoxifylline and rolipram. A suitable TNF antagonist that can prevent or inhibit TNF synthesis and/or TNF release also includes A2b adenosine receptor enhancers and A2b adenosine receptor agonists (e.g., 5'-(N-cyclopropyl)-carboxamidoadenosine, 5'-N-

20 ethylcarboxamidoadenosine, cyclohexyladenosine and R-N<sup>6</sup>-phenyl-2-propyladenosine). See, for example, Jacobson (GB 2 289 218 A), the teachings of which are entirely incorporated herein by reference. A suitable TNF

25 signalling.

#### Anti-TNF Antibodies

Anti-TNF antibodies useful in the methods of the present invention include monoclonal, chimeric, humanized, resurfaced and recombinant antibodies and fragments thereof

30 which are characterized by high affinity binding to TNF and low toxicity (including human anti-murine antibody (HAMA) and/or human anti-chimeric antibody (HACA) response). In particular, an antibody where the individual components, such as the variable region, constant region and framework,



individually and/or collectively possess low immunogenicity is useful in the present invention. The antibodies which can be used in the invention are characterized by their ability to treat patients for extended periods with good to  
5 excellent alleviation of symptoms and low toxicity. Low immunogenicity and/or high affinity, as well as other undefined properties, may contribute to the therapeutic results achieved.

10 An example of a high affinity monoclonal antibody useful in the methods of the present invention is murine monoclonal antibody (mAb) A2 and antibodies which will competitively inhibit *in vivo* the binding to human TNF $\alpha$  of anti-TNF $\alpha$  murine mAb A2 or an antibody having substantially the same specific binding characteristics, as well as  
15 fragments and regions thereof. Murine monoclonal antibody A2 and chimeric derivatives thereof are described in U.S. Application No. 08/192,093 (filed February 4, 1994), U.S. Application No. 08/192,102 (filed February 4, 1994), U.S. Application No. 08/192,861 (filed February 4, 1994), U.S. Application No. 08/324,799 (filed October 18, 1994), and  
20 Le, J. et al., International Publication No. WO 92/16553 (published October 1, 1992), which references are entirely incorporated herein by reference. A second example of a high affinity monoclonal antibody useful in the methods of  
25 the present invention is murine mAb 195 and antibodies which will competitively inhibit *in vivo* the binding to human TNF $\alpha$  of anti-TNF $\alpha$  murine 195 or an antibody having substantially the same specific binding characteristics, as well as fragments and regions thereof. Other high affinity  
30 monoclonal antibodies useful in the methods of the present invention include murine mAb 114 and murine mAb 199 and antibodies which will competitively inhibit *in vivo* the binding to human TNF $\alpha$  of anti-TNF $\alpha$  murine mAb 114 or mAb 199 or an antibody having substantially the same specific  
35 binding characteristics of mAb 114 or mAb 199, as well as

SECRET  
B-1  
C-1

U.S. Patent No. 5,656,272  
U.S. Patent No. 5,698,193  
ACI

fragments and regions thereof. Murine monoclonal antibodies 114, 195 and 199 and the method for producing them are described by Möller, A. et al. (*Cytokine* 2(3):162-169 (1990)), the teachings of which are entirely

5 incorporated herein by reference. Preferred methods for determining mAb specificity and affinity by competitive inhibition can be found in Harlow, et al., *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1988); Colligan et al., eds.,  
10 *Current Protocols in Immunology*, Greene Publishing Assoc. and Wiley Interscience, New York (1992, 1993); Kozbor et al., *Immunol. Today* 4:72-79 (1983); Ausubel et al., eds. *Current Protocols in Molecular Biology*, Wiley Interscience, New York (1987, 1992, 1993); and Muller, *Meth. Enzymol.*  
15 92:589-601 (1983), which references are entirely incorporated herein by reference.

Additional examples of monoclonal anti-TNF antibodies that can be used in the present invention are described in the art (see, e.g., Rathjen et al., International  
20 Publication No. WO 91/02078 (published February 21, 1991); Rubin et al., EPO Patent Publication 0218868 (published April 22, 1987); Yone et al., EPO Patent Publication 0288088 (October 26, 1988); Liang, et al., *Biochem. Biophys. Res. Comm.* 137:847-854 (1986); Meager, et al.,  
25 *Hybridoma* 6:305-311 (1987); Fendly et al., *Hybridoma* 6:359-369 (1987); Bringman, et al., *Hybridoma* 6:489-507 (1987); Hirai, et al., *J. Immunol. Meth.* 96:57-62 (1987); Moller, et al., *Cytokine* 2:162-169 (1990), which references are entirely incorporated herein by reference).

30 Chimeric antibodies are immunoglobulin molecules characterized by two or more segments or portions derived from different animal species. Generally, the variable region of the chimeric antibody is derived from a non-human mammalian antibody, such as a murine mAb, and the  
35 immunoglobulin constant region is derived from a human

immunoglobulin molecule. Preferably, a variable region with low immunogenicity is selected and combined with a human constant region which also has low immunogenicity, the combination also preferably having low immunogenicity.

5 "Low" immunogenicity is defined herein as raising significant HACA or HAMA responses in less than about 75%, or preferably less than about 50% of the patients treated and/or raising low titres in the patient treated (less than about 300, preferably less than about 100 measured with a  
10 double antigen enzyme immunoassay) (Elliott *et al.*, *Lancet* 344:1125-1127 (1994), incorporated herein by reference).

As used herein, the term "chimeric antibody" includes monovalent, divalent or polyvalent immunoglobulins. A monovalent chimeric antibody is a dimer (HL)) formed by a  
15 chimeric H chain associated through disulfide bridges with a chimeric L chain. A divalent chimeric antibody is a tetramer (H2L2) formed by two HL dimers associated through at least one disulfide bridge. A polyvalent chimeric antibody can also be produced, for example, by employing a  
20 CH region that aggregates (e.g., from an IgM H chain, or  $\mu$  chain).

Antibodies comprise individual heavy (H) and/or light (L) immunoglobulin chains. A chimeric H chain comprises an antigen binding region derived from the H chain of a  
25 non-human antibody specific for TNF, which is linked to at least a portion of a human H chain C region (CH), such as CH1 or CH2. A chimeric L chain comprises an antigen binding region derived from the L chain of a non-human antibody specific for TNF, linked to at least a portion of  
30 a human L chain C region (CL).

Chimeric antibodies and methods for their production have been described in the art (Morrison *et al.*, *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984); Boulianne *et al.*, *Nature* 312:643-646 (1984); Neuberger *et al.*, *Nature*  
35 314:268-270 (1985); Taniguchi *et al.*, European Patent

Application 171496 (published February 19, 1985); Morrison  
et al., European Patent Application 173494 (published March  
5, 1986); Neuberger et al., PCT Application WO 86/01533,  
(published March 13, 1986); Kudo et al., European Patent  
5 Application 184187 (published June 11, 1986); Morrison et  
al., European Patent Application 173494 (published March 5,  
1986); Sahagan et al., *J. Immunol.* 137:1066-1074 (1986);  
Robinson et al., International Publication No.  
PCT/US86/02269 (published 7 May 1987); Liu et al., *Proc.*  
10 *Natl. Acad. Sci. USA* 84:3439-3443 (1987); Sun et al., *Proc.*  
*Natl. Acad. Sci. USA* 84:214-218 (1987); Better et al.,  
*Science* 240:1041-1043 (1988); and Harlow and Lane,  
*Antibodies: A Laboratory Manual*, Cold Spring Harbor  
Laboratory, New York, 1988). These references are entirely  
15 incorporated herein by reference.

The anti-TNF chimeric antibody can comprise, for  
example, two light chains and two heavy chains, each of the  
chains comprising at least part of a human constant region  
and at least part of a variable (V) region of non-human  
20 origin having specificity to human TNF, said antibody  
binding with high affinity to an inhibiting and/or  
neutralizing epitope of human TNF, such as the antibody  
cA2. The antibody also includes a fragment or a derivative  
of such an antibody, such as one or more portions of the  
25 antibody chain, such as the heavy chain constant or  
variable regions, or the light chain constant or variable  
regions.

Humanizing and resurfacing the antibody can further  
reduce the immunogenicity of the antibody. See, for  
30 example, Winter (U.S. Patent No. 5,225,539 and EP 239,400  
B1), Padlan et al. (EP 519,596 A1) and Pedersen et al. (EP  
592,106 A1). These references are incorporated herein by  
reference.

Preferred antibodies useful in the methods of the  
35 present invention are high affinity human-murine chimeric

5

10

25

30

35

Ins.  
C2B

1.8x10<sup>9</sup>M<sup>-1</sup>. Preferred methods for determining mAb specificity and affinity by competitive inhibition can be found in Harlow, et al., *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1988; Colligan et al., eds., *Current Protocols in Immunology*, Greene Publishing Assoc. and Wiley Interscience, New York, (1992, 1993); Kozbor et al., *Immunol. Today* 4:72-79 (1983); Ausubel et al., eds. *Current Protocols in Molecular Biology*, Wiley Interscience, New York (1987, 1992, 1993); and Muller, *Meth. Enzymol.* 92:589-601 (1983), which references are entirely incorporated herein by reference.

As used herein, the term "antigen binding region" refers to that portion of an antibody molecule which contains the amino acid residues that interact with an antigen and confer on the antibody its specificity and affinity for the antigen. The antibody region includes the "framework" amino acid residues necessary to maintain the proper conformation of the antigen-binding residues. Generally, the antigen binding region will be of murine origin. In other embodiments, the antigen binding region can be derived from other animal species, such as sheep, rabbit, rat or hamster. Preferred sources for the DNA encoding such a non-human antibody include cell lines which produce antibody, preferably hybrid cell lines commonly known as hybridomas. In one embodiment, a preferred hybridoma is the A2 hybridoma cell line.

An "antigen" is a molecule or a portion of a molecule capable of being bound by an antibody which is additionally capable of inducing an animal to produce antibody capable of selectively binding to an epitope of that antigen. An antigen can have one or more than one epitope.

The term "epitope" is meant to refer to that portion of the antigen capable of being recognized by and bound by an antibody at one or more of the antibody's antigen

binding region. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and have specific three dimensional structural characteristics as well as specific charge characteristics. By "inhibiting and/or neutralizing epitope" is intended an epitope, which, when bound by an antibody, results in loss of biological activity of the molecule containing the epitope, *in vivo* or *in vitro*, more preferably *in vivo*, including binding of TNF to a TNF receptor. Epitopes of TNF have been identified within amino acids 1 to about 20, about 56 to about 77, about 108 to about 127 and about 138 to about 149. Preferably, the antibody binds to an epitope comprising at least about 5 amino acids of TNF within TNF residues from about 87 to about 107, about 59 to about 80 or a combination thereof. Generally, epitopes include at least about 5 amino acids and less than about 22 amino acids embracing or overlapping one or more of these regions.

For example, epitopes of TNF which are recognized by, and/or binds with anti-TNF activity, an antibody, and fragments, and variable regions thereof, include:

- 59-80: Tyr-Ser-Gln-Val-Leu-Phe-Lys-Gly-Gln-Gly-Cys-Pro-Ser-Thr-His-Val-Leu-Leu-Thr-His-Thr-Ile (SEQ ID NO:1); and/or
- 87-108: Tyr-Gln-Thr-Lys-Val-Asn-Leu-Leu-Ser-Ala-Ile-Lys-Ser-Pro-Cys-Gln-Arg-Glu-Thr-Pro-Glu-Gly (SEQ ID NO:2).

The anti-TNF antibodies, and fragments, and variable regions thereof, that are recognized by, and/or binds with anti-TNF activity, these epitopes block the action of TNF $\alpha$  without binding to the putative receptor binding locus as presented by Eck and Sprang (*J. Biol. Chem.* 264(29): 17595-17605 (1989) (amino acids 11-13, 37-42, 49-57 and

155-157 of hTNF $\alpha$ ). Rathjen et al., International Publication W0 91/02078 (published February 21, 1991), incorporated herein by reference, discloses TNF ligands which can bind additional epitopes of TNF.

## 5 Antibody Production Using Hybridomas

The techniques to raise antibodies to small peptide sequences that recognize and bind to those sequences in the free or conjugated form or when presented as a native sequence in the context of a large protein are well known in the art. Such antibodies can be produced by hybridoma or recombinant techniques known in the art.

Murine antibodies which can be used in the preparation of the antibodies useful in the methods of the present invention have also been described in Rubin et al., EP0218868 (published April 22, 1987); Yone et al., EP0288088 (published October 26, 1988); Liang, et al., *Biochem. Biophys. Res. Comm.* 137:847-854 (1986); Meager, et al., *Hybridoma* 6:305-311 (1987); Fendly et al., *Hybridoma* 6:359-369 (1987); Bringman, et al., *Hybridoma* 6:489-507 (1987); Hirai, et al., *J. Immunol. Meth.* 96:57-62 (1987); Möller, et al., *Cytokine* 2:162-169 (1990).

The cell fusions are accomplished by standard procedures well known to those skilled in the field of immunology. Fusion partner cell lines and methods for fusing and selecting hybridomas and screening for mAbs are well known in the art. See, e.g., Ausubel *infra*, Harlow *infra*, and Colligan *infra*, the contents of which references are incorporated entirely herein by reference.

The TNF $\alpha$ -specific murine mAb useful in the methods of the present invention can be produced in large quantities by injecting hybridoma or transfectoma cells secreting the antibody into the peritoneal cavity of mice and, after appropriate time, harvesting the ascites fluid which contains a high titer of the mAb, and isolating the mAb





Fab, Fab', F(ab')<sub>2</sub> and Fv. These fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and can have less non-specific tissue binding than an intact antibody (Wahl et al., *J. Nucl. Med.*

- 5 24:316-325 (1983)). These fragments are produced from intact antibodies using methods well known in the art, for example by proteolytic cleavage with enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')<sub>2</sub> fragments).

10 **Recombinant Expression of Anti-TNF Antibodies**

Recombinant and/or chimeric murine-human or human-human antibodies that inhibit TNF can be produced using known techniques based on the teachings provided in U.S. Application No. 08/192,093 (filed February 4, 1994),

- 15 U.S. Application No. 08/192,102 (filed February 4, 1994), <sup>now U.S. Patent No. 5,656,272</sup>  
U.S. Application No. 08/192,861 (filed February 4, 1994), <sup>AC3</sup>  
U.S. Application Serial No. 08/324,799 (filed on October 18, 1994, <sup>now U.S. Patent No. 5,698,195</sup> and Le, J. et al., International Publication No. WO 92/16553 (published October 1, 1992), which references

- 20 are entirely incorporated herein by reference. See, e.g., Ausubel et al., eds. *Current Protocols in Molecular Biology*, Wiley Interscience, N.Y. (1987, 1992, 1993); and Sambrook et al. *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (1989), the contents of  
25 which are entirely incorporated herein by reference. See also, e.g., Knight, D.M., et al., *Mol. Immunol* 30:1443-1453 (1993); and Siegel, S.A., et al., *Cytokine* 7(1):15-25 (1995), the contents of which are entirely incorporated herein by reference.

- 30 The DNA encoding an anti-TNF antibody can be genomic DNA or cDNA which encodes at least one of the heavy chain constant region (Hc), the heavy chain variable region (Hc), the light chain variable region (Lv) and the light chain constant regions (Lc). A convenient alternative to the use

of chromosomal gene fragments as the source of DNA encoding the murine V region antigen-binding segment is the use of cDNA for the construction of chimeric immunoglobulin genes, e.g., as reported by Liu et al. (*Proc. Natl. Acad. Sci., USA* 84:3439 (1987) and *J. Immunology* 139:3521 (1987)), which references are entirely incorporated herein by reference. The use of cDNA requires that gene expression elements appropriate for the host cell be combined with the gene in order to achieve synthesis of the desired protein. The use of cDNA sequences is advantageous over genomic sequences (which contain introns), in that cDNA sequences can be expressed in bacteria or other hosts which lack appropriate RNA splicing systems. An example of such a preparation is set forth below.

Because the genetic code is degenerate, more than one codon can be used to encode a particular amino acid. Using the genetic code, one or more different oligonucleotides can be identified, each of which would be capable of encoding the amino acid. The probability that a particular oligonucleotide will, in fact, constitute the actual XXX-encoding sequence can be estimated by considering abnormal base pairing relationships and the frequency with which a particular codon is actually used (to encode a particular amino acid) in eukaryotic or prokaryotic cells expressing an anti-TNF antibody or fragment. Such "codon usage rules" are disclosed by Lathe, et al., *J. Mol. Biol.* 183:1-12 (1985). Using the "codon usage rules" of Lathe, a single oligonucleotide, or a set of oligonucleotides, that contains a theoretical "most probable" nucleotide sequence capable of encoding anti-TNF variable or constant region sequences is identified.

Although occasionally an amino acid sequence can be encoded by only a single oligonucleotide, frequently the amino acid sequence can be encoded by any of a set of similar oligonucleotides. Importantly, whereas all of the

members of this set contain oligonucleotides which are capable of encoding the peptide fragment and, thus, potentially contain the same oligonucleotide sequence as the gene which encodes the peptide fragment, only one member of the set contains the nucleotide sequence that is identical to the nucleotide sequence of the gene. Because this member is present within the set, and is capable of hybridizing to DNA even in the presence of the other members of the set, it is possible to employ the unfractionated set of oligonucleotides in the same manner in which one would employ a single oligonucleotide to clone the gene that encodes the protein.

The oligonucleotide, or set of oligonucleotides, containing the theoretical "most probable" sequence capable of encoding an anti-TNF antibody or fragment including a variable or constant region is used to identify the sequence of a complementary oligonucleotide or set of oligonucleotides which is capable of hybridizing to the "most probable" sequence, or set of sequences. An oligonucleotide containing such a complementary sequence can be employed as a probe to identify and isolate the variable or constant region anti-TNF gene (Sambrook *et al.*, *infra*).

A suitable oligonucleotide, or set of oligonucleotides, which is capable of encoding a fragment of the variable or constant anti-TNF region (or which is complementary to such an oligonucleotide, or set of oligonucleotides) is identified (using the above-described procedure), synthesized, and hybridized by means well known in the art, against a DNA or, more preferably, a cDNA preparation derived from cells which are capable of expressing anti-TNF antibodies or variable or constant regions thereof. Single stranded oligonucleotide molecules complementary to the "most probable" variable or constant anti-TNF region peptide coding sequences can be synthesized

using procedures which are well known to those of ordinary skill in the art (Belagaje, et al., *J. Biol. Chem.* 254:5765-5780 (1979); Maniatis, et al., *In: Molecular Mechanisms in the Control of Gene Expression*, Nierlich, et al., Eds., Acad. Press, NY (1976); Wu, et al., *Prog. Nucl. Acid Res. Molec. Biol.* 21:101-141 (1978); Khorana, *Science* 203:614-625 (1979)). Additionally, DNA synthesis can be achieved through the use of automated synthesizers. Techniques of nucleic acid hybridization are disclosed by 5  
10 Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (1989); and by Haynes, et al., in: *Nucleic Acid Hybridization, A Practical Approach*, IRL Press, Washington, DC (1985), which references are entirely incorporated herein by reference. 15  
15 Techniques such as, or similar to, those described above have successfully enabled the cloning of genes for human aldehyde dehydrogenases (Hsu, et al., *Proc. Natl. Acad. Sci. USA* 82:3771-3775 (1985)), fibronectin (Suzuki, et al., *Bur. Mol. Biol. Organ. J.* 4:2519-2524 (1985)), the human 20  
20 estrogen receptor gene (Walter, et al., *Proc. Natl. Acad. Sci. USA* 82:7889-7893 (1985)), tissue-type plasminogen activator (Pennica, et al., *Nature* 301:214-221 (1983)) and human placental alkaline phosphatase complementary DNA (Keun, et al., *Proc. Natl. Acad. Sci. USA* 82:8715-8719 25  
25 (1985)).

In an alternative way of cloning a polynucleotide encoding an anti-TNF variable or constant region, a library of expression vectors is prepared by cloning DNA or, more preferably, cDNA (from a cell capable of expressing an 30  
30 anti-TNF antibody or variable or constant region) into an expression vector. The library is then screened for members capable of expressing a protein which competitively inhibits the binding of an anti-TNF antibody, such as A2 or cA2, and which has a nucleotide sequence that is capable of 35  
35 encoding polypeptides that have the same amino acid

sequence as anti-TNF antibodies or fragments thereof. In this embodiment, DNA, or more preferably cDNA, is extracted and purified from a cell which is capable of expressing an anti-TNF antibody or fragment. The purified cDNA is

5 fragmentized (by shearing, endonuclease digestion, etc.) to produce a pool of DNA or cDNA fragments. DNA or cDNA fragments from this pool are then cloned into an expression vector in order to produce a genomic library of expression vectors whose members each contain a unique cloned DNA or

10 cDNA fragment such as in a lambda phage library, expression in prokaryotic cell (e.g., bacteria) or eukaryotic cells, (e.g., mammalian, yeast, insect or, fungus). See, e.g., Ausubel, *infra*, Harlow, *infra*, Colligan, *infra*; Nyyssonen et al. *Bio/Technology* 11:591-595 (1993); Marks et al.,

15 *Bio/Technology* 11:1145-1149 (October 1993). Once nucleic acid encoding such variable or constant anti-TNF regions is isolated, the nucleic acid can be appropriately expressed in a host cell, along with other constant or variable heavy or light chain encoding nucleic acid, in order to provide

20 recombinant monoclonal antibodies that bind TNF with inhibitory activity. Such antibodies preferably include a murine or human anti-TNF variable region which contains a framework residue having complementarity determining residues which are responsible for antigen binding.

25 Human genes which encode the constant (C) regions of the chimeric antibodies, fragments and regions of the present invention can be derived from a human fetal liver library, by known methods. Human C region genes can be derived from any human cell including those which express

30 and produce human immunoglobulins. The human CH region can be derived from any of the known classes or isotypes of human H chains, including gamma,  $\mu$ ,  $\alpha$ ,  $\delta$  or  $\epsilon$ , and subtypes thereof, such as G1, G2, G3 and G4. Since the H chain isotype is responsible for the various effector functions

35 of an antibody, the choice of CH region will be guided by

the desired effector functions, such as complement fixation, or activity in antibody-dependent cellular cytotoxicity (ADCC). Preferably, the CH region is derived from gamma 1 (IgG1), gamma 3 (IgG3), gamma 4 (IgG4), or  $\mu$  (IgM). The human CL region can be derived from either human L chain isotype, kappa or lambda.

Genes encoding human immunoglobulin C regions are obtained from human cells by standard cloning techniques (Sambrook, et al. (*Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, New York (1989) and Ausubel et al., eds., *Current Protocols in Molecular Biology* (1987-1993)). Human C region genes are readily available from known clones containing genes representing the two classes of L chains, the five classes of H chains and subclasses thereof. Chimeric antibody fragments, such as  $F(ab')_2$  and Fab, can be prepared by designing a chimeric H chain gene which is appropriately truncated. For example, a chimeric gene encoding an H chain portion of an  $F(ab')_2$  fragment would include DNA sequences encoding the CH1 domain and hinge region of the H chain, followed by a translational stop codon to yield the truncated molecule.

Generally, the murine, human and chimeric antibodies, fragments and regions are produced by cloning DNA segments encoding the H and L chain antigen-binding regions of a TNF-specific antibody, and joining these DNA segments to DNA segments encoding CH and CL regions, respectively, to produce murine, human or chimeric immunoglobulin-encoding genes. Thus, in a preferred embodiment, a fused chimeric gene is created which comprises a first DNA segment that encodes at least the antigen-binding region of non-human origin, such as a functionally rearranged V region with joining (J) segment, linked to a second DNA segment encoding at least a part of a human C region.

Therefore, cDNA encoding the antibody V and C regions

and the method of producing a chimeric antibody can involve several steps, outlined below:

- 5 1. isolation of messenger RNA (mRNA) from the cell line producing an anti-TNF antibody and from optional additional antibodies supplying heavy and light constant regions; cloning and cDNA production therefrom;
- 10 2. preparation of a full length cDNA library from purified mRNA from which the appropriate V and/or C region gene segments of the L and H chain genes can be: (i) identified with appropriate probes, (ii) sequenced, and (iii) made compatible with a C or V gene segment from another antibody for a chimeric antibody;
- 15 3. Construction of complete H or L chain coding sequences by linkage of the cloned specific V region gene segments to cloned C region gene, as described above;
- 20 4. Expression and production of L and H chains in selected hosts, including prokaryotic and eukaryotic cells to provide murine-murine, human-murine, human-human or human-murine antibodies.

One common feature of all immunoglobulin H and L chain  
25 genes and their encoded mRNAs is the J region. H and L chain J regions have different sequences, but a high degree of sequence homology exists (greater than 80%) among each group, especially near the C region. This homology is exploited in this method and consensus sequences of H and L  
30 chain J regions can be used to design oligonucleotides for



use as primers for introducing useful restriction sites into the J region for subsequent linkage of V region segments to human C region segments.

C region cDNA vectors prepared from human cells can be  
5 modified by site-directed mutagenesis to place a  
restriction site at the analogous position in the human  
sequence. For example, one can clone the complete human  
kappa chain C (Ck) region and the complete human gamma-1 C  
region (C gamma-1). In this case, the alternative method  
10 based upon genomic C region clones as the source for C  
region vectors would not allow these genes to be expressed  
in bacterial systems where enzymes needed to remove  
intervening sequences are absent. Cloned V region segments  
are excised and ligated to L or H chain C region vectors.  
15 Alternatively, the human C gamma-1 region can be modified  
by introducing a termination codon thereby generating a  
gene sequence which encodes the H chain portion of an Fab  
molecule. The coding sequences with linked V and C regions  
are then transferred into appropriate expression vehicles  
20 for expression in appropriate hosts, prokaryotic or  
eukaryotic.

Two coding DNA sequences are said to be "operably  
linked" if the linkage results in a continuously  
translatable sequence without alteration or interruption of  
25 the triplet reading frame. A DNA coding sequence is  
operably linked to a gene expression element if the linkage  
results in the proper function of that gene expression  
element to result in expression of the coding sequence.

Expression vehicles include plasmids or other vectors.  
30 Preferred among these are vehicles carrying a functionally  
complete human CH or CL chain sequence having appropriate  
restriction sites engineered so that any VH or VL chain  
sequence with appropriate cohesive ends can be easily  
inserted therein. Human CH or CL chain sequence-containing  
35 vehicles thus serve as intermediates for the expression of

any desired complete H or L chain in any appropriate host.

A chimeric antibody, such as a mouse-human or human-human, will typically be synthesized from genes driven by the chromosomal gene promoters native to the mouse H and L chain V regions used in the constructs; splicing usually occurs between the splice donor site in the mouse J region and the splice acceptor site preceding the human C region and also at the splice regions that occur within the human C region; polyadenylation and transcription termination occur at native chromosomal sites downstream of the human coding regions.

A nucleic acid sequence encoding at least one anti-TNF antibody fragment may be recombined with vector DNA in accordance with conventional techniques, including blunt-ended or staggered-ended termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases. Techniques for such manipulations are disclosed, e.g., by Ausubel, *supra*, Sambrook, *supra*, entirely incorporated herein by reference, and are well known in the art.

A nucleic acid molecule, such as DNA, is "capable of expressing" a polypeptide if it contains nucleotide sequences which contain transcriptional and translational regulatory information and such sequences are "operably linked" to nucleotide sequences which encode the polypeptide. An operable linkage is a linkage in which the regulatory DNA sequences and the DNA sequence sought to be expressed are connected in such a way as to permit gene expression as anti-TNF peptides or antibody fragments in recoverable amounts. The precise nature of the regulatory regions needed for gene expression may vary from organism to organism and is well known in the analogous art. See, e.g., Sambrook et al., *Molecular Cloning: A Laboratory*

Manual, Cold Spring Harbor Laboratory Press (1989); and Ausubel, eds. *Current Protocols in Molecular Biology*, Wiley Interscience (1987, 1993).

Many vector systems are available for the expression  
5 of cloned anti-TNF peptide H and L chain genes in mammalian  
cells (see Glover, ed., *DNA Cloning*, Vol. II, pp143-238,  
IRL Press, 1985). Different approaches can be followed to  
obtain complete H2L2 antibodies. It is possible to  
co-express H and L chains in the same cells to achieve  
10 intracellular association and linkage of H and L chains  
into complete tetrameric H2L2 antibodies. The  
co-expression can occur by using either the same or  
different plasmids in the same host. Genes for both H and  
L chains can be placed into the same plasmid, which is then  
15 transfected into cells, thereby selecting directly for  
cells that express both chains. Alternatively, cells can  
be transfected first with a plasmid encoding one chain, for  
example the L chain, followed by transfection of the  
resulting cell line with an H chain plasmid containing a  
20 second selectable marker. Cell lines producing H2L2  
molecules via either route could be transfected with  
plasmids encoding additional copies of peptides, H, L, or H  
plus L chains in conjunction with additional selectable  
markers to generate cell lines with enhanced properties,  
25 such as higher production of assembled H2L2 antibody  
molecules or enhanced stability of the transfected cell  
lines.

### Receptor Molecules

Receptor molecules useful in the methods of the  
30 present invention are those that bind TNF with high  
affinity (see, e.g., Feldmann et al., International  
Publication No. WO 92/07076 (published April 30, 1992),  
incorporated herein by reference) and possess low  
immunogenicity. In particular, the 55 kDa (p55 TNF-R) and

the 75 kDa (p75 TNF-R) TNF cell surface receptors are useful in the present invention. Truncated forms of these receptors, comprising the extracellular domains (ECD) of the receptors or functional portions thereof, are also  
5 useful in the present invention. Truncated forms of the TNF receptors, comprising the ECD, have been detected in urine and serum as 30 kDa and 40 kDa TNF inhibitory binding proteins (Engelmann, H., et al., *J. Biol. Chem.* 265:1531-1536 (1990)). TNF receptor multimeric molecules and TNF  
10 immunoreceptor fusion molecules, and derivatives and fragments or portions thereof, are additional examples of receptor molecules which are useful in the methods of the present invention. The receptor molecules which can be used in the invention are characterized by their ability to  
15 treat patients for extended periods with good to excellent alleviation of symptoms and low toxicity. Low immunogenicity and/or high affinity, as well as other undefined properties, may contribute to the therapeutic results achieved.

20 TNF receptor multimeric molecules useful in the present invention comprise all or a functional portion of the ECD of two or more TNF receptors linked via one or more polypeptide linkers. The multimeric molecules can further comprise a signal peptide of a secreted protein to direct  
25 expression of the multimeric molecule. These multimeric molecules and methods for their production have been described in U.S. Application No. 08/437,533 (filed May 9, 1995), the content of which is entirely incorporated herein by reference.

30 TNF immunoreceptor fusion molecules useful in the methods of the present invention comprise at least one portion of one or more immunoglobulin molecules and all or a functional portion of one or more TNF receptors. These immunoreceptor fusion molecules can be assembled as  
35 monomers, or hetero- or homo-multimers. The immunoreceptor

fusion molecules can also be monovalent or multivalent.

5 TNF immunoreceptor fusion molecules and methods for  
their production have been described in the art (Lesslauer  
et al., *Eur. J. Immunol.* 21:2883-2886 (1991); Ashkenazi et  
al., *Proc. Natl. Acad. Sci. USA* 88:10535-10539 (1991);  
10 Peppel et al., *J. Exp. Med.* 174:1483-1489 (1991); Kolls et  
al., *Proc. Natl. Acad. Sci. USA* 91:215-219 (1994); Butler  
et al. *Cytokine* 6(6):616-623 (1994); Baker et al., *Eur. J.*  
*Immunol.* 24:2040-2048 (1994); Beutler et al., U.S. Patent  
15 No. 5,447,851; and U.S. Patent Application No. 08/442,133  
(filed May 16, 1995)). These references are entirely  
incorporated herein by reference. Methods for producing  
immunoreceptor fusion molecules can also be found in Capon  
et al., U.S. Patent No. 5,116,964; Capon et al., U.S.  
20 Patent No. 5,225,538; and Capon et al., *Nature* 337:525-531  
(1989), which references are entirely incorporated herein  
by reference.

25 Derivatives, fragments, regions and functional  
portions of the receptor molecules functionally resemble  
the receptor molecules that can be used in the present  
invention (i.e., they bind TNF with high affinity and  
possess low immunogenicity). A functional equivalent or  
derivative of the receptor molecule refers to the portion  
of the receptor molecule, or the portion of the receptor  
30 molecule sequence which encodes the receptor molecule, that  
is of sufficient size and sequences to functionally  
resemble the receptor molecules that can be used in the  
present invention (i.e., bind TNF with high affinity and  
possess low immunogenicity). A functional equivalent of  
the receptor molecule also includes modified receptor  
35 molecules that functionally resemble the receptor molecules  
that can be used in the present invention (i.e., bind TNF  
with high affinity and possess low immunogenicity). For  
example, a functional equivalent of the receptor molecule  
can contain a "SILENT" codon or one or more amino acid

substitutions, deletions or additions (e.g., substitution of one acidic amino acid for another acidic amino acid; or substitution of one codon encoding the same or different hydrophobic amino acid for another codon encoding a hydrophobic amino acid). See Ausubel et al., *Current Protocols in Molecular Biology*, Greene Publishing Assoc. and Wiley-Interscience (1989).

### Administration

TNF antagonists can be administered to an individual in a variety of ways. The routes of administration include intradermal, transdermal (e.g., in slow release polymers), intramuscular, intraperitoneal, intravenous, subcutaneous, oral, epidural and intranasal routes. Any other therapeutically efficacious route of administration can be used, for example, infusion or bolus injection, absorption through epithelial or mucocutaneous linings, or by gene therapy wherein a DNA molecule encoding the TNF antagonist is administered to the patient, e.g., via a vector, which causes the TNF antagonist to be expressed and secreted at therapeutic levels *in vivo*. In addition, the TNF antagonists can be administered together with other components of biologically active agents, such as pharmaceutically acceptable surfactants (e.g., glycerides), excipients (e.g., lactose), carriers, diluents and vehicles. If desired, certain sweetening, flavoring and/or coloring agents can also be added. The TNF antagonists can be administered prophylactically or therapeutically to an individual prior to, simultaneously with or sequentially with other therapeutic regimens or agents (e.g., multiple drug regimens), in a therapeutically effective amount. TNF antagonists that are administered simultaneously with other therapeutic agents can be administered in the same or different compositions.

For parenteral (e.g., intravenous, subcutaneous,

intramuscular) administration, TNF antagonists can be formulated as a solution, suspension, emulsion or lyophilized powder in association with a pharmaceutically acceptable parenteral vehicle. Examples of such vehicles  
5 are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Liposomes and nonaqueous vehicles such as fixed oils can also be used. The vehicle or lyophilized powder can contain additives that maintain isotonicity (e.g., sodium chloride, mannitol) and chemical  
10 stability (e.g., buffers and preservatives). The formulation is sterilized by commonly used techniques.

Suitable pharmaceutical carriers are described in Remington's Pharmaceutical Sciences, A. Osol, a standard reference text in this field of art.

15 For example, a parenteral composition suitable for administration by injection is prepared by dissolving 1.5% by weight of active ingredient in 0.9% sodium chloride solution.

20 A "therapeutically effective amount" is such that when administered, the TNF antagonist results in inhibition of the biological activity of TNF, relative to the biological activity of TNF when a therapeutically effective amount of the antagonist is not administered.

The dosage administered to an individual will vary  
25 depending upon a variety of factors, including the pharmacodynamic characteristics of the particular antagonists, and its mode and route of administration; size, age, sex, health, body weight and diet of the recipient; nature and extent of symptoms of the disease or  
30 disorder being treated, kind of concurrent treatment, frequency of treatment, and the effect desired. *In vitro* and *in vivo* methods of determining the inhibition of TNF in an individual are well known to those of skill in the art. Such *in vitro* assays can include a TNF cytotoxicity assay  
35 (e.g., the WEHI assay or a radioimmunoassay, ELISA). *In*

vivo methods can include rodent lethality assays and/or primate pathology model systems (Mathison *et al.*, *J. Clin. Invest.*, 81: 1925-1937 (1988); Beutler *et al.*, *Science* 229: 869-871 (1985); Tracey *et al.*, *Nature* 330: 662-664 (1987); Shimamoto *et al.*, *Immunol. Lett.* 17: 311-318 (1988); Silva *et al.*, *J. Infect. Dis.* 162: 421-427 (1990); Opal *et al.*, *J. Infect. Dis.* 161: 1148-1152 (1990); Hinshaw *et al.*, *Circ. Shock* 30: 279-292 (1990)).

10 TNF antagonist can be administered in single or multiple doses depending upon factors such as nature and extent of symptoms, kind of concurrent treatment and the effect desired. Thus, other therapeutic regimens or agents can be used in conjunction with the methods of the present invention. Adjustment and manipulation of established  
15 dosage ranges are well within the ability of those skilled in the art.

Usually a daily dosage of active ingredient can be about 0.01 to 100 milligrams per kilogram of body weight. Ordinarily 1 to 40 milligrams per kilogram per day given in  
20 divided doses 1 to 6 times a day or in sustained release form is effective to obtain desired results. Second or subsequent administrations can be administered at a dosage which is the same, less than or greater than the initial or previous dose administered to the individual.

25 A second or subsequent administration is preferably during or immediately prior to relapse or a flare-up of the disease or symptoms of the disease. For example, second and subsequent administrations can be given between about one day to 30 weeks from the previous administration. Two,  
30 three, four or more total administrations can be delivered to the individual, as needed. The terms "reoccurrence", "flare-up" or "relapse" are defined to encompass the reappearance of one or more symptoms of the disease state.

Dosage forms (composition) suitable for internal  
35 administration generally contain from about 0.1 milligram



to about 500 milligrams of active ingredient per unit. In these pharmaceutical compositions the active ingredient will ordinarily be present in an amount of about 0.5-95% by weight based on the total weight of the composition.

- 5 The present invention will now be illustrated by the following example, which is not intended to be limiting in any way.

### EXAMPLE

#### METHODS

##### 10 Trial procedures

The selection of patients for this study and their treatment have been described in detail previously (Elliott et al., *Lancet* 344:1105-1110 (1994)). In brief, 73 patients meeting the revised ACR criteria for the diagnosis of RA (Arnett et al., *Arthritis Rheum.* 31:315-321 (1988)) were recruited from the clinics of 4 cooperating trial centers. All patients had active RA and evidence of erosive disease on X-rays of hands or feet. Patients taking disease modifying anti-rheumatic drugs were withdrawn from their therapy at least 4 weeks prior to study entry, but were permitted to continue taking low dose oral corticosteroids or non-steroidal anti-inflammatory drugs at stable dosage.

cA2 is a human/murine chimeric monoclonal antibody of IgG1 $\kappa$  isotype, with specificity for recombinant and natural human TNF $\alpha$ . Chimeric monoclonal antibody cA2 and the method for producing it is described in U.S. Application No. 08/192,093 (filed February 4, 1994), U.S. Application No. 08/192,102 (filed February 4, 1994), <sup>now U.S. Patent No. 5,656,272</sup> U.S. Application No. 08/192,861 (filed February 4, 1994), <sup>AC 4</sup> U.S. Application Serial No. 08/324,799 (filed on October 18, 1994, <sup>now U.S. Patent No. 5,698,195</sup> and Le, J. et al., International Publication No. WO 92/16553 (published October 1, 1992), which references are entirely

036627E-063636

A  
Clns  
C4

incorporated herein by reference. At entry to the study, patients were randomised to receive a single 2 hour infusion of either placebo (0.1% human serum albumin, 24 patients), low dose cA2 (1 mg/kg, 25 patients) or high dose cA2 (10 mg/kg, 24 patients) as an outpatient procedure. Patients were then followed using clinical and laboratory parameters for a period of 4 weeks. Patients, investigators and laboratory personnel were blinded as to the treatment administered.

10        Blood samples for measurements of cytokines and acute phase proteins were drawn prior to the infusion on day 0 and at the following times after completion of the infusion: 1 and 8 hours, 1 and 3 days, 1, 2, 3, 4, weeks. This resulted in the following mean collection times:  
15        0830, 1300, 2000 (on day 0); 1200, 1100 (on days 1 and 3); 1030, 1100, 1045, 1045 (on weeks 1-4). Blood was collected into sterile tubes, allowed to clot for 30 minutes and spun at room temperature for 20 minutes at 2500 rpm. Serum was aliquoted into plastic tubes and stored at -70°C until  
20        assayed. Plasma was prepared from EDTA blood and handled similarly.

#### Laboratory Measurements

Measurement of circulating cytokines and acute phase proteins was made using commercially available assays, according to the manufacturers' directions. All samples from a given patient were assayed together, to reduce inter-assay variability.

#### Cytokines

30        IL-6 was measured by an enzyme amplified sensitivity immunoassay technique, based on an oligoclonal detection system (Medgenix Diagnostics, Brussels, Belgium). Briefly, serum samples were incubated in microtiter plates precoated with a cocktail of monoclonal antibodies to IL-6 and bound

cytokine was detected by the addition of complementary monoclonal antibodies to IL-6, conjugated to horse radish peroxidase. Optical density values obtained at 450 nm were compared to those obtained for a series of standards  
5 covering the range 10-2000 pg/ml.

#### Acute Phase Proteins

CRP was measured by fluorescent polarisation immunoassay using the TDX system (Abbot Diagnostics, Maidenhead, UK). The system works by comparing the  
10 polarisation value obtained for a given sample to a pre-calibrated standard value. Each assay was validated by the inclusion of control sera containing known quantities of CRP.

SAA was measured by a solid phase ELISA (Biosource  
15 Inc., Camarillo, CA). Diluted serum samples were incubated in microtiter plates precoated with a monoclonal antibody to SAA, together with a second complementary enzyme conjugated anti-SAA monoclonal antibody. Optical density values were compared to those obtained for a series of  
20 standards covering the range 0-300 ng/ml. Levels greater than 300 ng/ml were measured by repeating the assay using further sample dilutions.

Haptoglobin was measured using radial immunodiffusion (Behring, Hounslow, UK). EDTA plasma was placed into a  
25 well cut into a gel containing antibodies to haptoglobin. After 48 hours the diameter of the resulting precipitin rings was measured and the concentration compared to a pre-determined concentration table. The assay was validated by the inclusion in each assay of control sera of known  
30 concentration.

Fibrinogen was measured using radial immunodiffusion (Behring, Hounslow, UK). EDTA plasma was placed into a well cut into a gel containing antibodies to fibrinogen. After 18 hours the diameter of the resulting precipitin

ring was measured and the concentration compared to a pre-determined concentration table. The assay was validated by the inclusion in each assay of control sera of known concentration.

## 5 Statistics

Samples giving values below the assay detection limit were ascribed a value half the relevant detection limit prior to analysis. Data are expressed as median, interquartile range. Diurnal variation in IL-6 was assessed in the placebo group using the Wilcoxon signed rank test. Analysis of variance on the van der Waerden normal scores was used to compare baseline values of IL-6, CRP and SAA as well as comparison of changes from baseline at each post-treatment point. The model included terms for both investigational site and treatment group. Significant differences were further tested by Dunnett's comparison to the placebo group. The Mann Whitney U test was used to compare the haptoglobin and fibrinogen data for the placebo and high dose cA2 groups. Comparison between the percent reductions in IL-6, CRP and SAA in the high dose cA2 group was made using the Kruskal-Wallis test. Associations between parameters were defined using Spearman's rank correlation coefficient ( $\rho$ ). No adjustment was made for multiplicity of time points or laboratory parameters. Analyses were performed on a VAX computer using SAS and on a PowerMacintosh computer using Minitab.

## RESULTS

### X Effect of cA2 on Haptoglobin and Fibrinogen

In order to further define the biological effects of cA2, plasma levels of haptoglobin and fibrinogen were measured as representative of later elements within the acute phase response (Table). The data are expressed as median, interquartile range, with the number of patients

shown in brackets.

Table Plasma Haptoglobin and Fibrinogen

	<u>Placebo</u>	<u>10 mg/kg cA2</u>
<u>Haptoglobin (g/l)</u>		
Day 0	3.4, 3.0-4.3 [20]	3.3, 2.7-3.7 [15]
Day 28	3.4, 3.0-4.3 [20]	2.5, 1.5-3.2 [15] p<0.001
<u>Fibrinogen (g/l)</u>		
Day 0	3.8, 3.4-5.0 [20]	4.1, 3.2-4.7 [14]
Day 28	4.0, 3.3-5.4 [20]	2.8, 2.2-3.1 [14] p<0.002

Normal ranges: haptoglobin 0.7-3.8 g/l  
fibrinogen 1.8-3.5 g/l.

Pre-treatment haptoglobin and fibrinogen levels were similar in the placebo and high dose cA2 treatment groups. Placebo-treated patients showed no significant change in either measure, while the high dose cA2 group showed a significant reduction in both measures by week 4 (p<0.001, p<0.002, haptoglobin and fibrinogen respectively). (p values assess significance of the change from pre-treatment values in the cA2 group compared with change in the placebo group, by Mann Whitney U test).

#### 20 Effect of cA2 on Circulating IL-6

In defining the biological effects of cA2, in addition to plasma levels of fibrinogen and haptoglobin, circulating IL-6 was also measured. IL-6 was detectable in all but 4 of the 72 pre-treatment sera tested. The median, interquartile range pre-treatment circulating IL-6 levels for the 3 treatment groups were 125, 56-209 pg/ml, N=24; 130, 57-225 pg/ml, N=24; 114, 78-188 pg/ml, N=24 (placebo,

low and high dose cA2 respectively,  $p > 0.05$ , normal range  $< 10$  pg/ml).

The changes in circulating IL-6 following treatment are shown in Figures 1A and 1B (\* indicates  $p < 0.05$ ,  
5 \*\* indicates  $p < 0.01$ , \*\*\* indicates  $p < 0.001$  compared with placebo, by ANOVA). Each point represents the median change from day 0 values in up to 24 patients, with interquartile ranges omitted for clarity. Patients were  
10 treated on the morning of day 0 with a single, 2 hour infusion of either placebo (circle), 1 mg/kg cA2 (triangle) or 10 mg/kg cA2 (square).

IL-6 levels showed significant reductions in the placebo group at the 1300 and 2000 hours time points on day 0 ( $p < 0.001$ ,  $p = 0.002$  respectively), with partial recovery by  
15 day 1 (Figure 1A). Patients treated with cA2 showed even more marked reductions in circulating IL-6 at 1300 hours on day 0 and continuing decline thereafter, reaching significance compared with placebo by day 1 ( $p < 0.01$ ,  $p < 0.001$ , low and high dose cA2 respectively). In Figure  
20 1B, changes in serum IL-6 over the longer term are displayed. The highly significant falls in serum IL-6 seen at day 1 were maintained for the duration of the study in patients receiving high dose cA2 but there was a partial loss of effect in patients treated with low dose cA2 by  
25 week 4 (Figure 1B).

The changes in median IL-6 values were reflected in the individual patient responses for IL-6. Of the 24 low dose cA2 patients tested, 22 had elevated IL-6 values pre-treatment and 14 of the 22 (64%) showed normalisation of  
30 values from day 1. Similarly, 23 of 24 high dose cA2 patients had elevated circulating IL-6 pre-treatment, of whom 16 (70%) normalised from day 1.

The detection of elevated circulating IL-6 in the patients is consistent with previous reports, which showed  
35 the presence of IL-6 in the majority of RA sera, although

at lower concentrations than found in matched synovial fluid samples (Houssiau *et al.*, *Arthritis Rheum.* 31:784-788 (1988); Swaak *et al.*, *Scand. J. Rheumatol.* 17:469-474 (1988); Arvidson *et al.*, *Ann. Rheum. Dis.* 53:521-524 (1994)). The reductions in circulating IL-6 in placebo treated patients during the course of days 0 and 1 are also consistent with the recognised diurnal variation in this cytokine in patients with RA (Arvidson *et al.*, *Ann. Rheum. Dis.* 53:521-524 (1994)). The reductions in circulating IL-6 in cA2-treated patients were even more marked than in the placebo group, reaching significance from day 1. These findings support an earlier, preliminary report of reductions in circulating IL-6 in patients treated in the open label trial of cA2 (Elliott *et al.*, *Arthritis Rheum.* 36:1681-1690 (1993)) and concur with the results of an open label trial of cA2 in active, refractory Crohn's disease (Van Dullemen *et al.*, *Gastroenterology* 109:129-135 (1995)). Although all but two of the Crohn's patients studied had baseline circulating IL-6 levels within the normal range, a significant fall was seen after cA2 treatment. The results described herein provide in vivo evidence that TNF $\alpha$  is in part regulatory for IL-6 production in RA synovial tissue. In vitro findings also demonstrated that TNF $\alpha$  is in part regulatory for IL-6 production in RA synovial tissue.

#### 25 Effect of cA2 on CRP and SAA

To further define the biological effects of cA2, circulating CRP and SAA levels were measured. The changes in circulating CRP values in this study have been reported previously (Elliott *et al.*, *Lancet* 344:1105-1110 (1994)). In brief, patients treated with placebo showed no significant change in CRP values, while those treated with either low or high dose cA2 showed large and highly significant reductions, evident as early as day 1 post-treatment and reaching their maximal extent by day 7. The

median, interquartile range pre-treatment CRP values were: 56, 33-70 mg/l; 58, 34-84 mg/l; 65, 28-94 mg/l (placebo, low and high dose cA2 groups, respectively,  $p > 0.05$ , normal range  $< 10$  mg/l) and by day 7 the equivalent values were:

- 5 56, 31-72 mg/l; 21, 16-25 mg/l; 18, 13-35 mg/l ( $p < 0.001$  for both low and high dose cA2, compared with placebo).

Changes in circulating SAA following treatment with cA2 are shown in Figure 2 (\* indicates  $p < 0.05$ , \*\* indicates  $p < 0.01$ , \*\*\* indicates  $p < 0.001$  compared with placebo, by

- 10 ANOVA). Each point represents the median change from day 0 values in up to 24 patients, with interquartile ranges omitted for clarity. Patients were treated on the morning of day 0 with a single, 2 hour infusion of either placebo (circle), 1 mg/kg cA2 (triangle) or 10 mg/kg cA2 (square).

- 15 Pre-treatment circulating SAA showed a tendency to higher values in the 1 mg/kg cA2 group than in other treatment groups (335, 62-750 mg/l, N=24; 591, 188-1130 mg/l, N=25; 378, 180-935 mg/l, N=24; placebo, low and high dose cA2 respectively; normal range  $< 10$  mg/l), although  
20 this difference was not statistically significant. No significant changes in SAA levels were seen following treatment in the placebo group (Figure 2), while those patients treated with either low or high dose cA2 showed large and highly significant reductions. These changes  
25 were less rapid than those seen for IL-6, with maximal improvements delayed until day 3 (Figure 2). A trend towards a loss of response in SAA for patients treated with low dose cA2 was noted towards the end of the study (Figure 2).

30 Association Between Circulating IL-6 and Acute Phase Proteins

Because IL-6 is the principal regulator of hepatic acute phase protein synthesis in vitro (Gauldie et al., *Proc. Natl. Acad. Sci. USA* 84:7251-7255 (1987); Baumann et



al., *Immunol. Today* 15:74-80 (1994)), the association between IL-6, two acute phase reactants, CRP and SAA, was tested in the patients. A comparison of the kinetics of change in each of these mediators following treatment with high dose cA2 is shown in Figure 3. Each point represents the median of values from 24 patients, expressed as a percentage of the pre-treatment values. Square = circulating IL-6, triangle = CRP, and X = SAA before (day 0) and after treatment with a single, 2 hour infusion of high dose cA2 (10mg/kg). \*\*\* indicates  $p < 0.001$ , comparing IL-6, CRP and SAA, by Kruskal-Wallis test.

By day 1, median circulating IL-6 values had fallen compared with pre-treatment values by 95%, whereas reductions in CRP (20%) and SAA (5%) were much more modest ( $p < 0.001$ ). Although the median values for the two acute phase proteins had fallen further by day 3, thereby narrowing the gap with IL-6, the reduction in IL-6 at this time was still significantly greater than for the acute phase proteins ( $p < 0.001$ ). This temporal relationship is consistent with regulation of CRP and SAA production by IL-6, but interpretation of the results is complicated by marked differences in the circulating half times of these molecules (Castell *et al.*, *Eur. J. Biochem.* 177:357-361 (1988); Vigushin *et al.*, *J. Clin. Invest.* 91:1351-1357 (1993)). The demonstration of significant correlations between both pre-treatment day 3 reductions in serum IL-6 and the corresponding values for the acute phase proteins provides further evidence for a relationship between these mediators.

A scatter graph comparing pre-treatment IL-6 and CRP levels in all 73 patients is shown in Figure 4A, indicating a moderate association between these variables ( $p = 0.55$ ,  $p < 0.002$ ). A similar association was found when comparing the reduction in circulating IL-6 by day 3 with the reduction in CRP over the same time period (Figure 4B;

p=0.59, p<0.002). (Each point in Figures 4A-4B represents an individual patient.) An association between circulating IL-6 levels and CRP in patients with inflammatory arthritis has been noted previously (Arvidson *et al.*, *Ann. Rheum.*

5 *Dis.* 53:521-524 (1994)). It is possible that other circulating cytokines with hepatocyte stimulating activity, such as leukemia inhibitory factor, may also contribute to acute phase protein synthesis in RA.

10 Less impressive, but still statistically significant associations were seen between circulating IL-6 and SAA (pre-treatment comparison: p=0.44, p<0.002; reduction by day 3 comparison: p=0.48, p<0.002). The strongest associations observed were between CRP and SAA (pre-treatment comparison: p=0.73, p<0.002; reduction by day 3:  
15 p=0.76, p<0.002). The results clearly show that TNF $\alpha$  blockade in the short term leads to normalisation of SAA levels in many patients.

20 Although the rapid response elements, CRP and SAA, are normally measured as markers of disease activity, rather than as pathophysiological agents in their own right, acute phase proteins may directly contribute to disease outcomes in RA. Prolonged, high level elevation in circulating SAA is associated with the development of secondary amyloidosis, a cause of renal failure and premature death  
25 in a small proportion of RA patients.

#### Equivalents

Those skilled in the art will know, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention  
30 described herein. These and all other equivalents are intended to be encompassed by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Elliott, Michael J.  
Maini, Ravinder N.  
Feldmann, Marc
- (ii) TITLE OF INVENTION: Methods of Preventing or Treating  
Cardiovascular, Cerebrovascular and Thrombotic Disorders  
with Tumor Necrosis Factor Antagonists
- (iii) NUMBER OF SEQUENCES: 2
- (iv) CORRESPONDENCE ADDRESS:  
(A) ADDRESSEE: Hamilton, Brook, Smith & Reynolds, P.C.  
(B) STREET: Two Militia Drive  
(C) CITY: Lexington  
(D) STATE: Massachusetts  
(E) COUNTRY: U.S.A.  
(F) ZIP: 02173-4799
- (v) COMPUTER READABLE FORM:  
(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:  
(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:  
(A) NAME: Brook, David E.  
(B) REGISTRATION NUMBER: 22,592  
(C) REFERENCE/DOCKET NUMBER: KIR96-01

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: 617-861-6240
- (B) TELEFAX: 617-861-9540

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Tyr	Ser	Gln	Val	Leu	Phe	Lys	Gly	Gln	Gly	Cys	Pro	Ser	Thr	His	Val
1				5				10						15	
Leu Leu Thr His Thr Ile															
20															

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

[illegible]

Arg Glu Thr Pro Glu Gly  
20

Arg Glu Thr Pro Glu Gly  
20

CLAIMS

What is claimed is:

1. A method for treating or preventing a cardiovascular disorder in an individual in need thereof comprising administering to the individual a therapeutically effective amount of a tumor necrosis factor antagonist.
2. A method of Claim 1 wherein the cardiovascular disorder is selected from the group consisting of: acute myocardial infarction, deep vein thrombosis and thrombophlebitis.
3. A method of Claim 2 wherein the cardiovascular disorder is acute myocardial infarction.
4. A method for treating or preventing a cerebrovascular disorder in an individual in need thereof comprising administering to the individual a therapeutically effective amount of a tumor necrosis factor antagonist.
5. A method of Claim 4 wherein the cerebrovascular disorder is stroke.
6. A method of treating or preventing a thrombotic disorder in an individual in need thereof comprising administering a therapeutically effective amount of a tumor necrosis factor antagonist to the individual.
7. A method of Claim 6 wherein the thrombotic disorder is selected from the group consisting of: a thromboembolic disorder, a ischemic event, stroke,

acute myocardial infarction, deep vein thrombosis and thrombophlebitis.

8. A method of Claim 7 wherein the tumor necrosis factor antagonist is an anti-tumor necrosis factor antibody or fragment thereof.
9. A method of Claim 8 wherein the antibody is selected from the group consisting of: a chimeric antibody, a humanized antibody and a resurfaced antibody or fragment thereof.
10. A method of Claim 9 wherein the antibody binds to one or more amino acids of hTNF $\alpha$  selected from the group consisting of about 87-108 and about 59-80.
11. A method of Claim 9 wherein the antibody binds to the epitope of A2.
12. A method of Claim 9 wherein the antibody is a chimeric antibody.
13. A method of Claim 12 wherein the antibody binds to one or more amino acids of hTNF $\alpha$  selected from the group consisting of about 87-108 and about 59-80.
14. A method of Claim 12 wherein the antibody binds to the epitope of cA2.
15. A method of Claim 14 wherein the antibody is cA2.
16. A method of Claim 7 wherein the tumor necrosis factor antagonist is a receptor molecule, derivative or a fragment thereof which binds to tumor necrosis factor.

17. A method of Claim 16 wherein the receptor molecule is selected from the group consisting of: p55 tumor necrosis factor receptor and p75 tumor necrosis factor receptor or functional portions thereof.
- 5 18. A method of Claim 16 wherein the receptor molecule is selected from the group consisting of: the extracellular domain of p55 tumor necrosis factor receptor and the extracellular domain of p75 tumor necrosis factor receptor.
- 10 19. A method of Claim 16 wherein the receptor molecule is a TNF receptor multimeric molecule or a functional portion thereof.
20. A method of Claim 19 wherein the tumor necrosis receptor multimeric molecule comprises all or a functional portion of two or more extracellular domains of tumor necrosis factor receptors linked via one or more polypeptide linkers.
- 15
21. A method of Claim 16 wherein the receptor molecule is an immunoreceptor fusion molecule or functional portion thereof.
- 20
22. A method of Claim 21 wherein the immunoreceptor fusion molecule comprises all or a functional portion of a tumor necrosis factor receptor and an immunoglobulin chain.
- 25 23. A method of Claim 7 wherein the tumor necrosis factor antagonist prevents or inhibits tumor necrosis factor synthesis or tumor necrosis factor release.
24. A method of Claim 23 wherein the tumor necrosis factor
- all  
av





- [illegible]

gl  
WB

consisting of about 87-108 and about 59-80.

33. A method of Claim 32 wherein the antibody binds to the epitope of A2.
34. A method of Claim 31 wherein the antibody is a chimeric antibody.
35. A method of Claim 34 wherein the antibody binds to one or more amino acids of hTNF $\alpha$  selected from the group consisting of about 87-108 and about 59-80.
36. A method of Claim 34 wherein the antibody binds to the epitope of cA2.
37. A method of Claim 36 wherein the antibody is cA2.
38. A method of Claim 29 wherein the tumor necrosis factor antagonist is a receptor molecule, derivative or a fragment thereof which binds to tumor necrosis factor.
39. A method of Claim 38 wherein the receptor molecule is selected from the group consisting of: p55 tumor necrosis factor receptor and p75 tumor necrosis factor receptor or functional portions thereof.
40. A method of Claim 38 wherein the receptor molecule is selected from the group consisting of: the extracellular domain of p55 tumor necrosis factor receptor and the extracellular domain of p75 tumor necrosis factor receptor.
41. A method of Claim 40 wherein the receptor molecule is a tumor necrosis factor receptor multimeric molecule.

42. A method of Claim 41 wherein the tumor necrosis factor receptor multimeric molecule comprises all or a functional portion of two or more extracellular domains of tumor necrosis factor receptors linked via one or more polypeptide linkers.
43. A method of Claim 40 wherein the receptor molecule is an immunoreceptor fusion molecule or functional portion thereof.
44. A method of Claim 43 wherein the immunoreceptor fusion molecule comprises all or a functional portion of a tumor necrosis factor receptor and an immunoglobulin chain.
45. A method of Claim 29 wherein the tumor necrosis factor antagonist prevents or inhibits tumor necrosis factor synthesis or tumor necrosis factor release.
46. A method of Claim 45 wherein the tumor necrosis factor antagonist is a phosphodiesterase inhibitor.
47. A method of Claim 46 wherein the phosphodiesterase inhibitor is selected from the group consisting of: pentoxifylline and rolipram.
48. A method of Claim 45 wherein the tumor necrosis factor antagonist is selected from the group consisting of: thalidomide and tenidap.
49. A method of Claim 45 wherein the tumor necrosis factor antagonist is selected from the group consisting of: a A2b adenosine receptor agonist and a A2b adenosine receptor enhancer.

- [illegible]

METHODS OF PREVENTING OR TREATING CARDIOVASCULAR,  
CEREBROVASCULAR AND THROMBOTIC DISORDERS  
WITH TUMOR NECROSIS FACTOR ANTAGONISTS

ABSTRACT OF THE DISCLOSURE

5        A method of treating or preventing a cardiovascular  
and/or a cerebrovascular disorder in an individual is  
disclosed. Also disclosed is a method for treating and/or  
preventing a thrombotic disorder in an individual. Further  
disclosed is a method of decreasing plasma fibrinogen in an  
10 individual.

Figure 1A

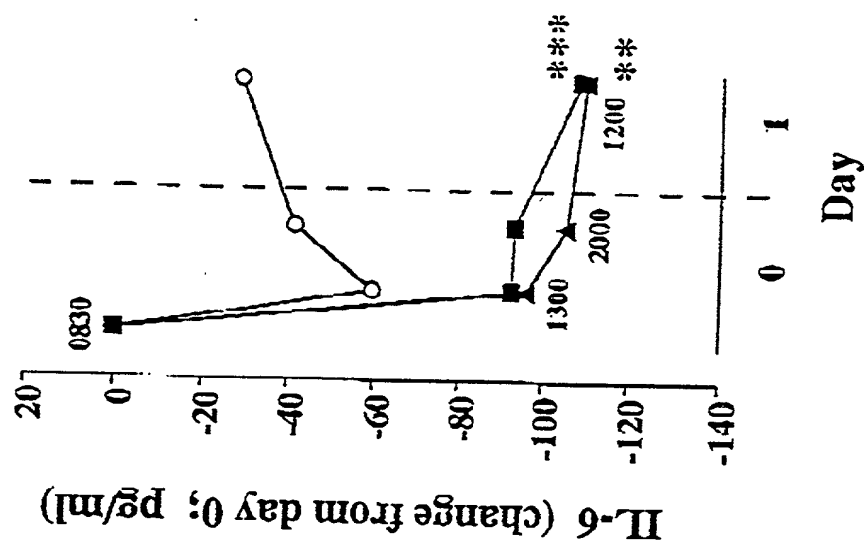
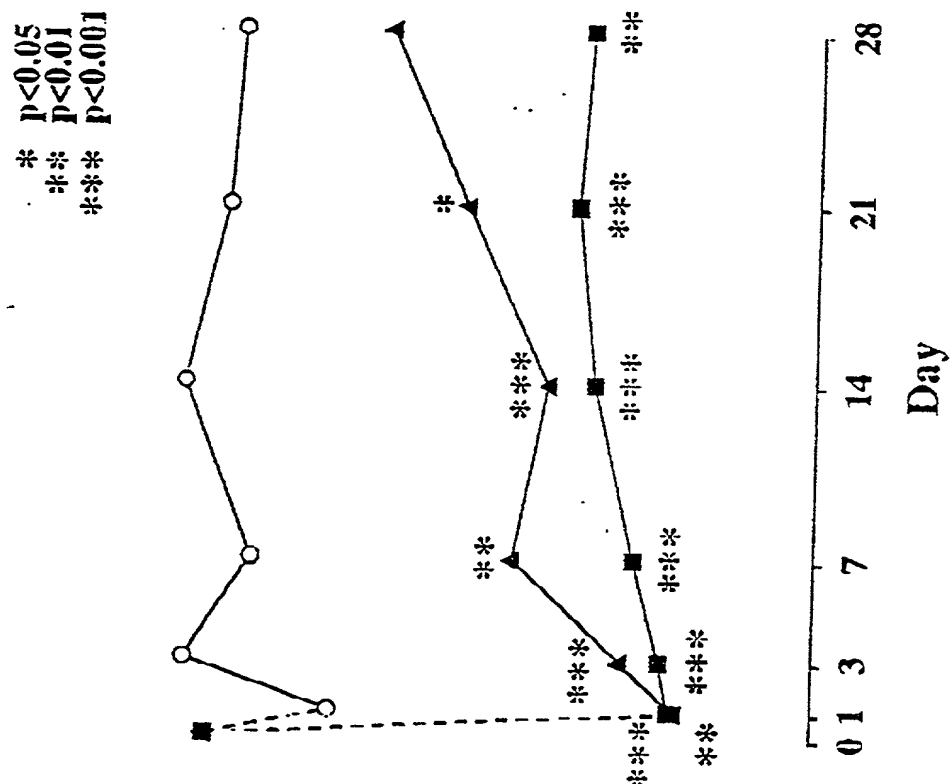


Figure 1B



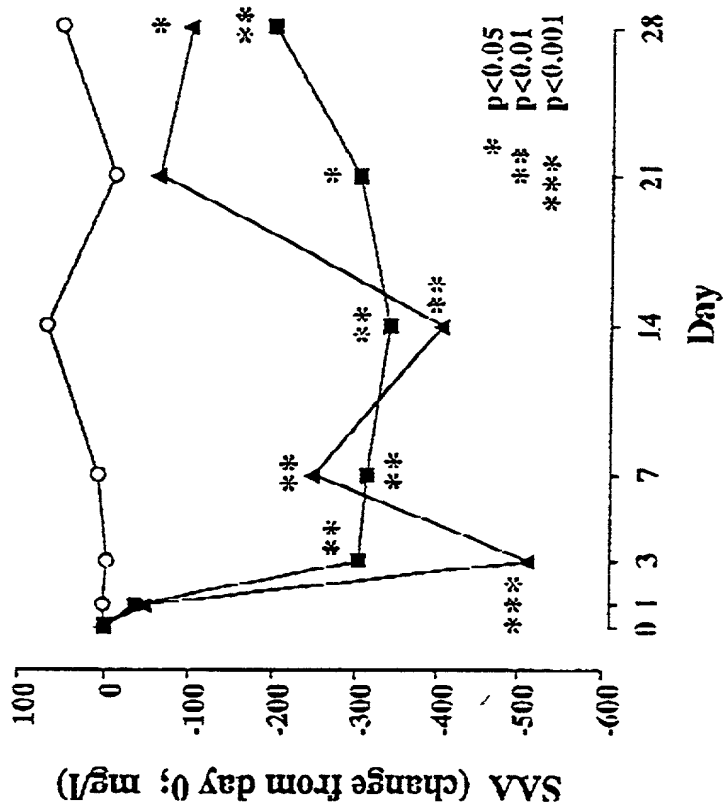


Figure 2

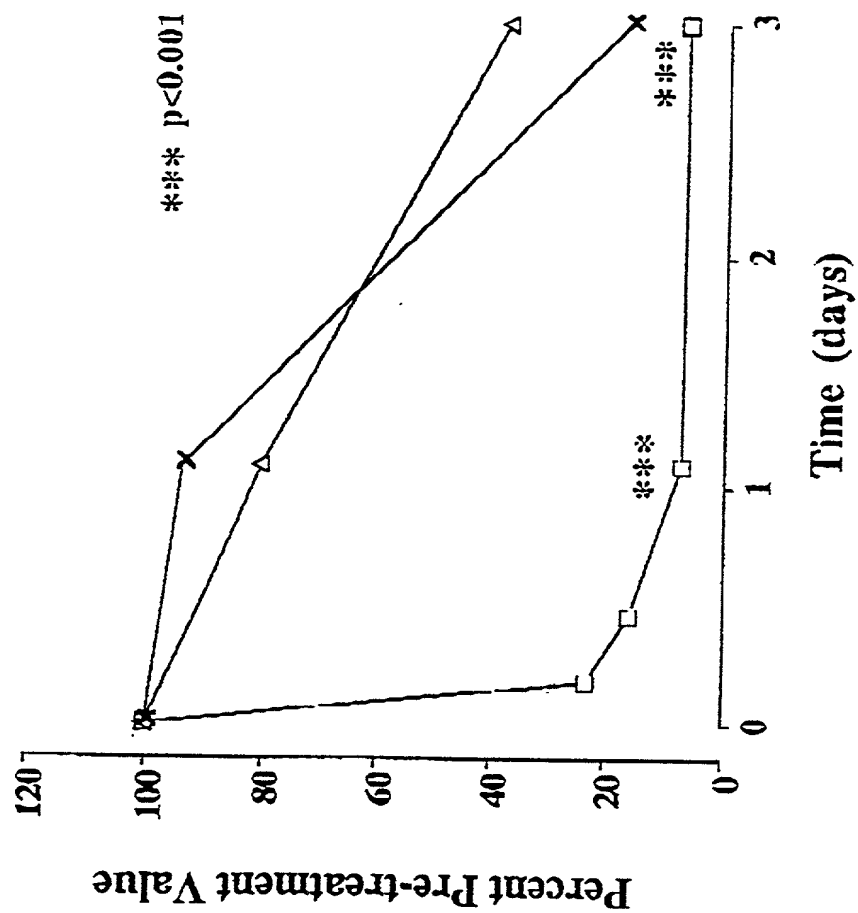
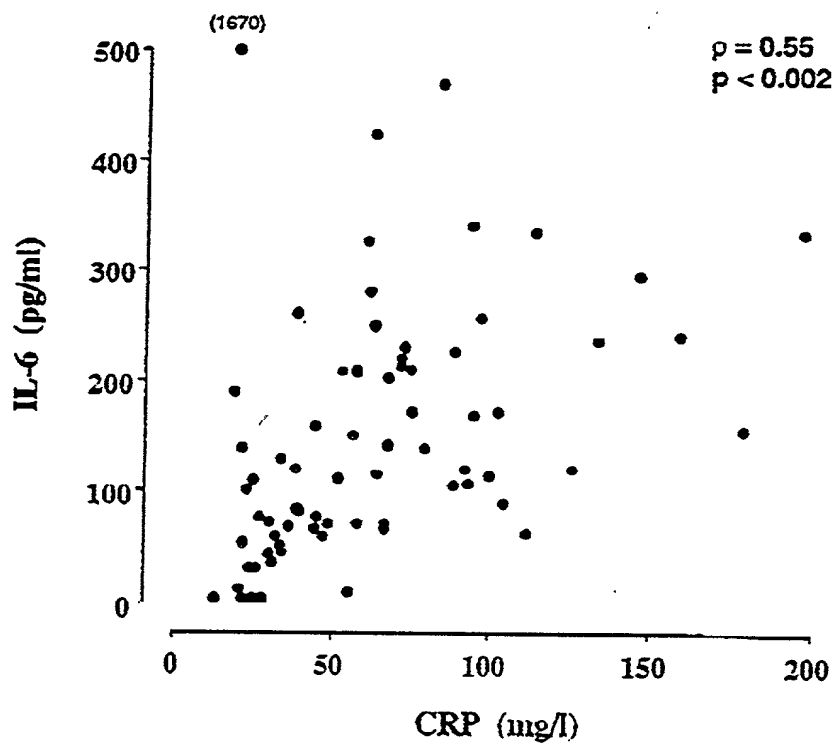


Figure 3



Figure 4 **A**Figure 4 **B**